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Published in:
Journal of Bacteriology

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
1995

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Buist, G., Kok, J., Leenhouts, K. J., Dabrowska, M., Venema, G., & Haandrikman, A. J. (1995). Molecular Cloning and Nucleotide Sequence of the Gene Encoding the Major Peptidoglycan Hydrolase of Lactococcus lactis, a Muramidase Needed for Cell Separation. *Journal of Bacteriology*, *177*(6), 1554-1563.

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JOURNAL OF BACTERIOLOGY, Mar. 1995, p. 1554–1563 Vol. 177, No. 6
0021-9193/95/$04.00+0
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Molecular Cloning and Nucleotide Sequence of the Gene Encoding the Major Peptidoglycan Hydrolase of *Lactococcus lactis*, a Muramidase Needed for Cell Separation

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Received 25 July 1994/Accepted 5 January 1995

A gene of *Lactococcus lactis* subsp. *cremoris* MG1363 encoding a peptidoglycan hydrolase was identified in a genomic library of the strain in pUC19 by screening *Escherichia coli* transformants for cell wall lysis activity on a medium containing autoclaved, lyophilized *Micrococcus lysodeikticus* cells. In cell extracts of *L. lactis* MG1363 and several halo-producing *E. coli* transformants, lytic bands of similar sizes were identified by denaturing sodium dodecyl sulfate (SDS)-polyacrylamide gels containing *L. lactis* or *M. lysodeikticus* cell walls. Of these clearing bands, corresponding to the presence of lytic enzymes with sizes of 46 and 41 kDa, the 41-kDa band was also present in the supernatant of an *L. lactis* culture. Deletion analysis of one of the recombinant plasmids showed that the information specifying lytic activity was contained within a 2,428-bp *Eco*RV-*Sau*3A fragment. Sequencing of part of this fragment revealed a gene (*acmA*) that could encode a polypeptide of 437 amino acid residues. The calculated molecular mass of AcmA (46,564 Da) corresponded to that of one of the lytic activities detected. Presumably, the enzyme is synthesized as a precursor protein which is processed by cleavage after the Ala at position 57, thus producing a mature protein with a size of 40,264 Da, which would correspond to the size of the enzyme whose lytic activity was present in culture supernatants of *L. lactis*. The N-terminal region of the mature protein showed 60% identity with the N-terminal region of the mature muramidase-2 of *Enterococcus hirae* and the autolysin of *Streptococcus faecalis*. Like the latter two enzymes, AcmA contains C-terminal repeated regions. In AcmA, these three repeats are separated by nonhomologous intervening sequences highly enriched in serine, threonine, and asparagine. Genes specifying identical activities were detected in various strains of *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* by the SDS-polyacrylamide gel electrophoresis detection assay and PCR experiments. By replacement recombination, an *acmA* deletion mutant which grew as long chains was constructed, indicating that AcmA is required for cell separation.

Bacteria produce several types of cell wall hydrolases, enzymes capable of hydrolyzing the peptidoglycan of the cell envelope. On the basis of their cleavage specificities, the enzymes are classified as *N*-acetylmuramidases (lysozymes), *N*-acetylglucosaminidases, *N*-acetylmuramyl-L-alanine amidases, endopeptidases, and transglycosylases (45). Cell wall hydrolases are thought to be involved in cell wall turnover, cell separation, competence for genetic transformation, formation of flagella, sporulation, and the lytic action of some general antibiotics (for reviews, see references 12, 39, and 52).

Strains of the starter bacterium *Lactococcus lactis* are of eminent economic importance because of their worldwide use in cheese making. It is generally believed that during cheese maturation, autolysis of the bacteria results in the release of intracellular proteolytic enzymes, such as a variety of peptidases which digest casein-borne peptides and, thus, may contribute to cheese flavor development (49).

Some data are available on the biochemistry of autolysis and autolytic activity in lactococci. Thus, Mou et al. (32) and Niskasaari (34) have shown that *L. lactis* subsp. *cremoris* shows maximal autolytic activity during exponential growth in media with a neutral pH. These investigators could detect only an *N*-acetylmuramidase, which was present in the cell wall and supernatant fraction; no endopeptidase or glucosaminidase activity was detectable. The lactococcal autolytic activity was inhibited by lipoteichoic acid and cardiolipin and activated by trypsin in in vitro experiments (34). Mou et al. (32) also showed that the cell walls of exponential-phase *L. lactis* subsp. *cremoris* cells autolyze most readily at the equatorial ring. McDonald (31) noted that in *L. lactis*, filament formation was associated with decreased autolysin activity, an observation later confirmed by Langsrud et al. (26). These data suggest that autolytic activity in *L. lactis* is involved in cell separation.

Although data on the biochemistry of lactococcal autolysins is gradually emerging, nothing is known about the genetic components governing autolysis in *L. lactis*. In this paper, we report the cloning, expression, and sequencing of the gene for the major peptidoglycan hydrolase from *L. lactis*. In addition, a deletion mutant was constructed to assess the function of the autolysin.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used in this study are listed in Table 1. *L. lactis* was grown in M17 broth (Difco, West Molesey, United Kingdom) or whey-based medium (11) at 30°C as standing cultures or on M17 agar, all of which were supplemented with 0.5% glucose.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant phenotype(s) or genotype(s)	Source or reference(s)
Strains		
L. lactis subsp. *cremoris*		
MG1363	Plasmid-free strain	14
MG1363::pINTAA-1	Em^r β-Gal^+, derivative of MG1363 with integrated pINTAA via portion 1	This work
MG1363::pINTAA-2	Em^r β-Gal^+, derivative of MG1363 with integrated pINTAA via portion 2	This work
MG1363*acmA*Δ*1*	Derivative of MG1363 containing 701-bp *Sac*I-*Spe*I chromosomal deletion in *acmA* gene	This work
AM1	Wild-type strain	13
HP	Wild-type strain	New Zealand Dairy Research Institute
L. lactis subsp. *lactis* IL1403	Plasmid-free strain	6
L. lactis subsp. *lactis* bv. diacetylactis 18-16S	Sm^r	15
E. coli		
NM522	*supE thi* Δ(*lac-proAB*) Δ*hsd5* (r_k^- m_k^-) [F′ *proAB lacI*^{q}*ZM15*]	Stratagene
EC1000	Km^r *glgB*, derivative of MC1000 containing *repA* gene of lactococcal plasmid pWV01	Laboratory collection
Plasmids		
pBluescript SK+	Ap^r	Stratagene
pUC19	Ap^r	54
pUK21	Km^r	48
pORI280	Em^r β-Gal^+, *ori*$^+$ of pWV01; integration vector which replicates in strains containing RepA	28, 30
pAL01	Ap^r pUC19 carrying 4,137-bp lactococcal chromosomal DNA insert with *acmA* gene	This work
pAL02	Ap^r, pAL01 with 507-bp *Eco*RV deletion	This work
pAL03	Km^r, pUK21 with 1,383-bp *Hin*dIII-*Sac*I insert of pAL01	This work
pAL04	Km^r, pUK21 with 1,467-bp *Hin*dIII-*Sac*I insert of pAL01	This work
pAL05	Ap^r, SK+ with 682-bp *Hin*dIII-*Spe*I fragment of pAL03	This work
pAL06	Ap^r, SK+ with 701-bp *Sac*I-*Spe*I fragment of pAL03	This work
pAL07	Ap^r, SK+ with 477-bp *Sca*I-*Cla*I fragment of pAL04	This work
pAL08	Ap^r, pAL01 with 16-bp *Sma*I-*Eco*RI deletion	This work
pAL09	Ap^r, pAL08 with 701-bp *Sac*I-*Spe*I deletion	This work
pINTAA	Em^r β-Gal^+, pORI280 with 2,247-bp *Eco*RV-*Hin*dIII fragment of pAL09	This work

Erythromycin (Boehringer GmbH, Mannheim, Germany) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Sigma Chemical Co., St. Louis, Mo.) were added to concentrations of 5 μg/ml and 0.008%, respectively. *Escherichia coli* was grown in TY (Difco Laboratories, Detroit, Mich.) medium at 37°C with vigorous agitation or on TY medium solidified with 1.5% (wt/vol) agar, containing 100 μg of ampicillin (Sigma) per ml, 50 μg of kanamicin (Boehringer) per ml, or 100 μg of erythromycin (Boehringer) per ml, when required. Isopropyl-β-D-thiogalactopyranoside (IPTG) and X-Gal (both from Sigma) were used at concentrations of 1 mM and 0.002% (wt/vol), respectively.

General DNA techniques and transformation. Molecular cloning techniques were performed essentially as described by Sambrook et al. (40). Restriction enzymes, the Klenow enzyme, T4 DNA polymerase, T4 DNA ligase, and deoxynucleotides were obtained from Boehringer Mannheim and were used according to the instructions of the supplier. Genomic DNA of *L. lactis* was isolated according to the method described by Leenhouts et al. (29), with one modification: cell pellets resuspended in a lysis solution with lysozyme were incubated at 55°C for 15 min (42). *E. coli* and *L. lactis* were transformed by electroporation by using a gene pulser (Bio-Rad Laboratories, Richmond, Calif.), as described by Zabarovsky and Winberg (56) and Holo and Nes (16), with the modifications suggested by Leenhouts and Venema (30), respectively.

Sample preparation for SDS-PAGE. After overnight growth, the optical density of the *L. lactis* cultures was measured in a Philips PU8720 UV/VIS scanning spectrophotometer (Pye Unicam Ltd., Cambridge, United Kingdom) at 600 nm. Five milliliters of the culture was subjected to centrifugation, and the supernatant fractions were dialyzed against several changes of demineralized water, lyophilized, and dissolved in 1 ml of denaturation buffer (3). The cell pellets were resuspended in 1 ml of denaturation buffer, and cell extracts were prepared as described by van de Guchte et al. (46). Cell extracts of *E. coli* strains were made accordingly after 2 ml of the overnight cultures was pelleted. The samples were boiled for 5 min and centrifuged before loading. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli (25), with the Protean II Minigel System (Bio-Rad). Prestained molecular weight markers were obtained from Bio-Rad and Pharmacia AB (Uppsala, Sweden).

Detection of lytic activity in SDS-polyacrylamide gels. Lytic activity was detected in situ by using SDS–12.5% (wt/vol) polyacrylamide gels containing 0.2% (wt/vol) autoclaved, lyophilized *Micrococcus lysodeikticus* ATCC 4698 cells (Sigma) or cell walls of *L. lactis* MG1363. Cell walls of *L. lactis* MG1363 were isolated by the method of Potvin et al. (38), with the following modification: after resuspension in 4% (wt/vol) SDS, the cells were disrupted as described by van de Guchte et al. (46). After electrophoresis, the gels were gently shaken at room temperature for 24 to 48 h in three to five changes of 100 ml of 25 mM Tris-HCl (pH 7) containing 1% (vol/vol) Triton X-100 to allow for protein renaturation (38). Bands of lytic activity were visualized by staining with 1% (wt/vol) methylene blue (Sigma) in 0.01% (wt/vol) KOH and subsequent destaining with demineralized water (18). SDS-polyacrylamide gels without cell walls were stained with Coomassie brilliant blue or by using a silver stain kit (Bio-Rad).

Construction of a chromosomal gene library of *L. lactis* MG1363 in *E. coli* and screening for lytic activity against *M. lysodeikticus*. A library of *L. lactis* MG1363 chromosomal DNA was constructed as described by Zabarovsky and Allikmets (55), with some modifications. Genomic DNA of *L. lactis* MG1363 was digested for 15 min with the appropriate amount of *Sau*3A to generate partial digests. After electrophoresis in ultrapure agarose (Pharmacia) of the chromosomal digest, fragments of 4 to 10 kb in size were isolated from the gel by electroelution. The isolated chromosomal DNA fragments, partially filled-in with dATP and dGTP, and pUC19 DNA linearized with *Sal*I, partially filled-in with dTTP and dCTP, were ligated, and the ligation mixture was used to electrotransform *E. coli* NM522. After electroporation, the cells were plated on TY plates containing

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0.2% (wt/vol) autoclaved, lyophilized *M. lysodeikticus* cells and placed at 37°C. After 2 days the plates were placed at room temperature and examined daily for the appearance of halos around the colonies.

Molecular cloning and DNA sequencing. For DNA sequencing, the two *Hin*dIII-*Sac*I fragments (1,467 and 1,383 bp, respectively) of pAL01 were subcloned into the *Hin*dIII and *Sac*I sites of pUK21, and the resulting plasmids were designated pAL04 and pAL03, respectively. The insert of pAL03 was subcloned into two fragments at the unique *Spe*I site. pAL05 was constructed by digestion of pAL03 with *Spe*I and *Hin*dIII and subcloning of the 682-bp fragment into the *Hin*dIII and *Spe*I sites of pBluescript SK+; the other part of the insert of pAL03 was subcloned as an *Spe*I-*Sac*I fragment (701 bp) into the same sites of pBluescript SK+. This subclone was named pAL06. The 352-bp *Sca*I-*Sac*I fragment of the insert of pAL04 was subcloned as an *Sca*I-*Cla*I fragment into the *Sma*I and *Cla*I sites of pBluescript SK+, resulting in pAL07.

Both strands of the inserts of the various subclones were sequenced by the dideoxy-chain termination method (41) with the T7 sequencing kit (Pharmacia) and double-stranded plasmid templates, according to the manufacturer's instructions, by using universal and reverse pUC primers. The sequence was completed with synthetic DNA primers. Primers were synthesized with a 381A DNA synthesizer (Applied Biosystems, Inc., Foster City, Calif.). DNA nucleotide and amino acid sequences were analyzed with the PC/GENE (version 6.7) sequence analysis program (IntelliGenetics, Inc., Geneva, Switzerland). Protein homology searches were carried out with the databases SWISSPROT (release 27) and ATLAS of protein and genomic sequences (March 1994) by means of the FASTA program (36).

Southern transfer, DNA hybridization, and PCR. After agarose gel electrophoresis, DNA was transferred to GeneScreen Plus membranes (NEN Research Products, Boston, Mass.) by the protocol of Southern, as modified by Chomczynski and Quasba (5). Probe labelling and hybridization were done with the ECL labelling and detection system according to the instructions of the manufacturer (Amersham International, Amersham, United Kingdom).

PCR was carried out with super *Taq* DNA polymerase according to the instructions of the manufacturer (HT Biotechnology Ltd., Cambridge, England) on chromosomal DNA with the primer combinations PALA-4 (5′-CTTCAACA GACAAGTCC) and PALA-14 (5′-GATAAATGATTCCAAGC), both located within the *acmA* gene (see Fig. 4) or PALA-19 (5′-CAAGGTTAAGTCCACG), which is located upstream of the insert of pINTAA, and BK05AL (5′-CAT TATTTGATTGGAGTT), which is located in the origin of replication of pORI280.

Construction of *acmA* deletion strain. A replacement recombination system developed by Leenhouts and Venema (28, 30) was used to replace *acmA* on the chromosome of *L. lactis* MG1363 with an *acmA* gene with an internal deletion. A unique *Sac*I site in *acmA* was obtained by cutting pAL01 with *Eco*RI and *Sma*I (see Fig. 3). The plasmid DNA was treated with a Klenow enzyme, ligated, and used to electrotransform *E. coli* NM522. The resulting plasmid, pAL08, was digested with *Sac*I and *Spe*I, treated with T4 DNA polymerase, ligated, and used to electrotransform *E. coli* NM522, giving plasmid pAL09. pAL09 was digested with *Bam*HI and *Eco*RV, and the DNA fragment containing the deleted *acmA* gene was subcloned into the *Bam*HI and *Sma*I sites of pORI280. The ligation mixture was used to electrotransform *E. coli* EC1000. The resulting integration plasmid, named pINTAA, was used to electrotransform *L. lactis* MG1363. Selection of the second crossover event was done as described by Leenhouts and Venema (30).

Nucleotide sequence accession number. The sequence identified in the study (see Fig. 4) has been assigned GenBank accession number U17696.

RESULTS

Analysis of peptidoglycan hydrolase activity of *L. lactis* MG1363. Biochemical analyses indicate that *L. lactis* has only *N*-acetylmuramidase activity (unpublished results) (32, 34). The peptidoglycan hydrolyzing activity of GM17 or whey-grown *L. lactis* MG1363 was examined by SDS-PAGE in the presence of either autoclaved, lyophilized *M. lysodeikticus* cells or isolated cell walls of *L. lactis* MG1363 as a substrate (Fig. 1). Eleven different clearing bands, corresponding to proteins ranging in size from 29 to 111 kDa, were detected in the supernatant fraction of the whey culture, but only three of these bands were detectable in this fraction of the GM17 culture when autoclaved *M. lysodeikticus* cells were used as a substrate. With cell walls of *L. lactis* MG1363, only three bands could be detected in the supernatant of the whey-grown cells and only one band, corresponding to an approximately 46-kDa protein, could be detected in the GM17 culture supernatant. In the cell extracts of the two cultures, only two clear bands were observed, corresponding to 41- and 46-kDa proteins, when *M. lysodeikticus* cells were used as a substrate. Only the smaller band was found in gels containing isolated lactococcal cell walls. All the activities could be detected when buffered Triton X-100 ranging from pH 3 to 10 was used in the renaturation, but the staining conditions were optimal after renaturation at pH 7. When samples were run on SDS-polyacrylamide gels without a substrate, protein bands corresponding to the clearing bands could not be detected in either the cell extract or the supernatant fraction after staining with Coomassie brilliant blue and subsequent silver staining (results not shown).

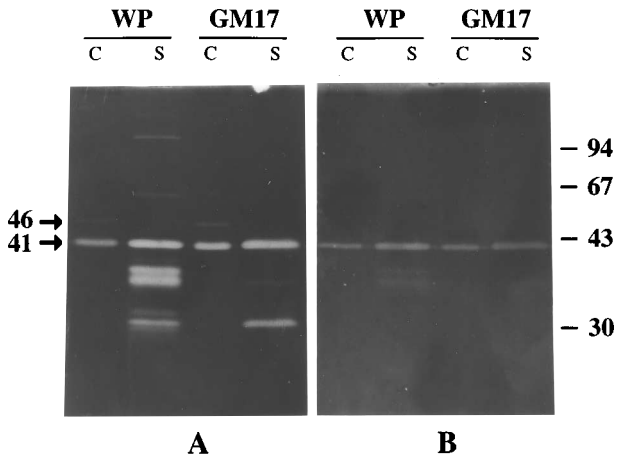

FIG. 1. Cell wall hydrolase activity of *L. lactis* MG1363 in renaturing SDS–12.5% PAGE. (A) Gel containing 0.2% (wt/vol) *M. lysodeikticus* autoclaved cells; (B) gel containing 0.2% (wt/vol) cell walls from *L. lactis* MG1363. Lanes: WP and GM17, fractions of cultures grown in whey-based medium and GM17, respectively; C, cell extract; S, supernatant fraction. The amount of sample loaded was equalized according to the optical density of the cultures. Molecular masses (in kilodaltons) of standard proteins are shown on the right, and the sizes (in kilodaltons) of two lytic bands discussed in the text are indicated on the left.

Peptidoglycan hydrolytic activity was also detected on GM17 agar plates in which *M. lysodeikticus* cells had been incorporated. Hydrolysis of the cell walls could be seen as zones of clearing around colonies of *L. lactis* MG1363 after 36 h of incubation at 30°C.

When a proteinase-positive strain of *L. lactis* MG1363 was examined for peptidoglycan hydrolase activity by renaturing SDS-PAGE, only a few small clearing bands were present in the supernatant fraction. The bands in the cell extract were the same as those seen in a proteinase-deficient strain (results not shown).

Cloning of the gene for peptidoglycan hydrolase of *L. lactis* MG1363. The average size of the insert of the *L. lactis* MG1363 genomic library in pUC19 was 6 kb. Of approximately 8,000 colonies tested, 13 produced a halo on TY plates containing *M. lysodeikticus* cells. Renaturing SDS-PAGE of cell extracts of these *E. coli* clones showed that all produced clearing bands with sizes of approximately 46 and 41 kDa, as shown in Fig. 2 for two selected clones. A 46-kDa band present in *E. coli* was present in the lactococcal cell extract only, whereas a 41-kDa band produced by *E. coli* was observed in both the cell extract and the supernatant fraction of *L. lactis*. Furthermore, several faster-migrating bands were visible when cell extracts of the positive *E. coli* clones were analyzed. The sizes of the inserts in pUC19 of the 13 *E. coli* clones ranged from 4.1 to 9 kb. Restriction enzyme analysis showed that the plasmids isolated from all lysin-producing colonies shared a 4.1-kb *Sau*3A insert. A clone containing only this 4.1-kb fragment showed growth problems. An overnight culture of the strain foamed heavily, and flocculation of cells was observed in the culture by visual

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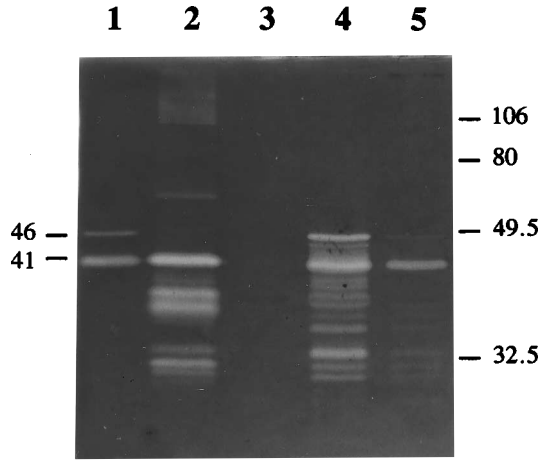

FIG. 2. Cell wall hydrolase activity in *E. coli* NM522 transformants by renaturing SDS–12.5% PAGE. The gel contained 0.2% (wt/vol) *M. lysodeikticus* autoclaved cells. Lanes: 1 and 2, cell extract and supernatant of *L. lactis* MG1363 grown in whey-based medium, respectively; 3, cell extract of *E. coli* NM522; 4 and 5, cell extracts of two independent *E. coli* NM522 transformants producing a halo on TY plates containing *M. lysodeikticus* autoclaved cells. Molecular masses (in kilodaltons) of standard proteins are shown on the right. Two lytic bands corresponding to sizes of 41 and 46 kDa are indicated on the left.

inspection and by light microscopy. This clone was selected for further experiments, and its plasmid was designated pAL01. A restriction map of the insert is presented in Fig. 3. Various restriction enzyme digests of chromosomal DNA of *L. lactis* MG1363 were analyzed by Southern hybridization with the 4.1-kb *Sau*3A insert of pAL01 as a probe. No rearrangements were found, as judged from a comparison of the restriction map of this fragment and the corresponding chromosomal DNA (results not shown).

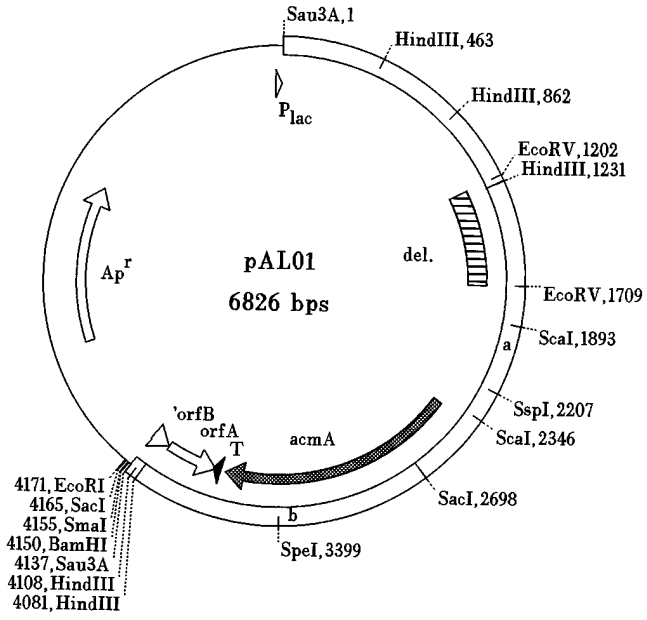

FIG. 3. Map of pAL01. The 4,137-bp *Sau*3A DNA fragment of the chromosome of *L. lactis* MG1363 is indicated by the open bar. pAL01 includes *acmA*, ORFA, and ORFB. T, putative terminator; P_{lac}, *lac* promoter and its direction of transcription (open arrowhead); del., *Eco*RV deletion made to construct pAL02; Ap^r, ampicillin resistance gene. The *Hin*dIII-*Sac*I fragments used to construct pAL04 and pAL03 are indicated by a and b, respectively. The genetic analysis of the 2,206-bp *Sau*3A-*Ssp*I fragment will be presented elsewhere (3a).

The 507-bp *Eco*RV fragment of pAL01 could be deleted without loss of any of the clearing bands. Because the activity band with the highest molecular mass present in the *E. coli*-(pAL02) cell extracts was 46 kDa and because the coding capacity of the smallest of the *Sau*3A-*Eco*RV fragments (1,201 bp) was insufficient to encode a 46-kDa protein, the 2,428-bp *Eco*RV-*Sau*3A fragment was subcloned and sequenced.

Nucleotide sequence of the lactococcal gene for peptidoglycan hydrolase. The two *Sac*I-*Hin*dIII fragments of pAL01 (Fig. 3, labelled a and b, respectively) were subcloned into pUK21, and several smaller subclones were made by using pBluescript SK+. The nucleotide sequence of the 1,930-bp *Ssp*I-*Sau*3A fragment of pAL01 (Fig. 4) shows that it contains two complete open reading frames (ORFs), with lengths of 1,311 (*acmA*) and 282 bp (ORFA). Upstream of ORFA, a part of a third ORF was found, suggesting that ORFA is the last ORF of an operon. Both ORFA and *acmA* are preceded by putative ribosome-binding sites complementary to the 3′ end of the lactococcal 16S rRNA (4) and have ΔG° values of −9.7 and −9.6 kcal/mol (−41 and −40 kJ/mol), respectively (44). Also, a possible −10 and −35 region with a spacing of 23 bp is present upstream of *acmA*. The putative −10 region is preceded by the sequence TGN, which is found in more than 40% of the lactococcal promoters analyzed so far (10). An inverted repeat with a ΔG° value of −16 kcal/mol (−70 kJ/mol) that may function as a rho-independent terminator is located downstream of *acmA* (37). The sequenced fragment has a G+C content of 36.2 mol%, which is in agreement with the G+C content determined for *L. lactis* (38.6 mol%) (33). The codon usage of *acmA* and ORFA is in agreement with that calculated by van de Guchte et al. (47) from several sequenced *L. lactis* genes.

Deduced amino acid sequences and homology comparisons. *acmA* could encode a protein with 437 amino acids with a deduced molecular weight of 46,564. The first 57 amino acids constitute a putative signal peptide (50), and a membrane-spanning domain was identified within this stretch of amino acids (Fig. 5). Cleavage of this putative signal peptide would result in a protein with a size of 40,264 Da. The entire protein has a predicted theoretical isoelectric point (pI) of 10.45. The protein without the putative signal sequence has a predicted pI of 10.12.

An identity of approximately 56% was found between the N-terminal part of AcmA (amino acids 65 to 220) and muramidase-2 of *Enterococcus hirae* (7) and the autolysin of *Streptococcus faecalis* (3) (Fig. 5A). A low degree of similarity was observed with the flagellar protein FlgJ of *Salmonella typhimurium* (20). From these data combined with the biochemical data available (unpublished data) (32, 34) and the results presented below, we conclude that we have cloned the gene encoding the major lysozyme (*N*-acetylmuramidase) of *L. lactis*. Therefore, the gene was called *acmA*.

In the C-terminal part of the protein, three repeated regions are present. The regions are 44 amino acids long and are separated by intervening sequences highly enriched for serine, threonine, and asparagine residues. The overall similarity between the repeated regions is approximately 75% (Fig. 6). At the DNA level, the homology is only 45%. The C-terminal repeats of AcmA have homology with the C-terminal repeated regions of the cell wall hydrolases of *E. hirae* and *S. faecalis*. The organization of these repeats in the three enzymes is schematically presented in Fig. 5B. This part of AcmA also shows homology with protein p60 of *Listeria monocytogenes*,

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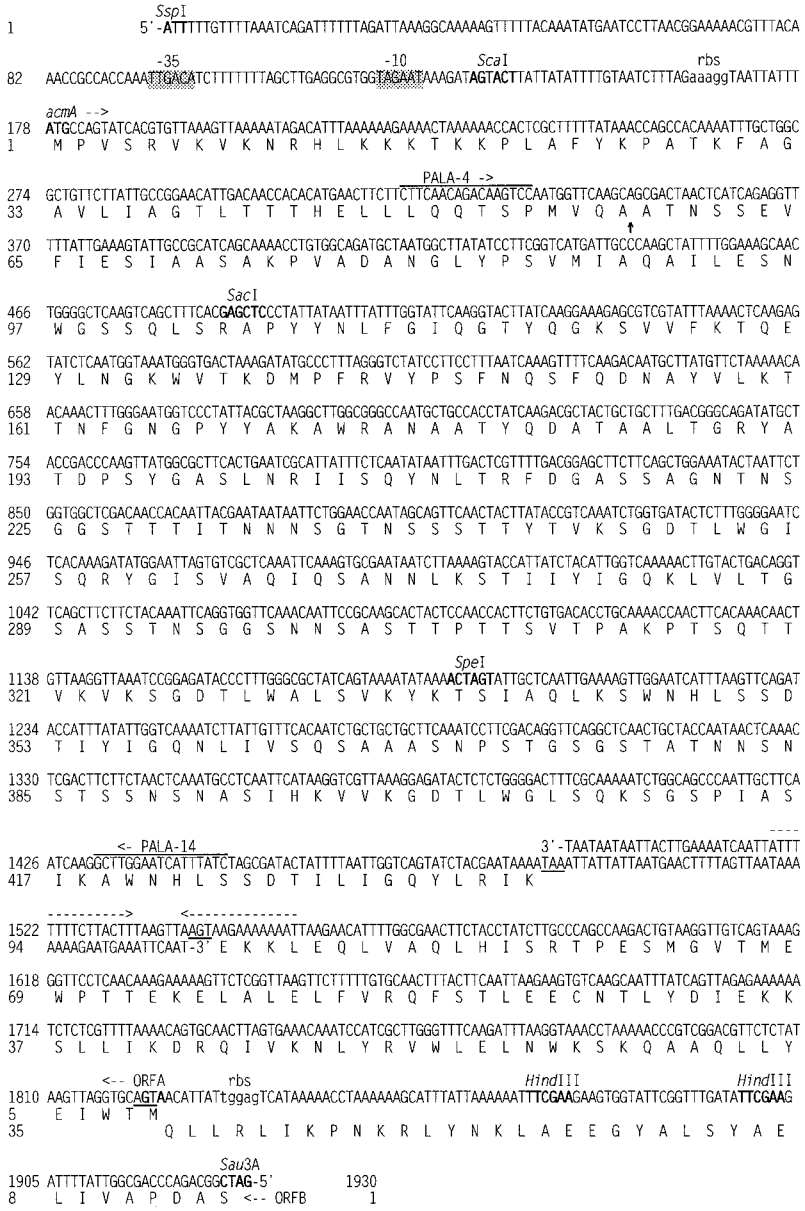

FIG. 4. Nucleotide sequence and deduced amino acid sequences of *acmA*, ORFA and part of ORFB of *L. lactis* MG1363. Putative ribosome-binding sites (rbs and lowercase letters), −10 and −35 sequences (shaded), start codons (boldface), and stop codons (underlined) are indicated. A possible transcriptional terminator is indicated by horizontal arrows above the sequence. The synthetic primers PALA-4 and PALA-14 used in the PCR experiment are overlined. The possible signal peptide cleavage site is indicated by a vertical arrow. A number of relevant restriction enzyme sites are also indicated. The intergenic region between *acmA* and ORFA is shown double stranded.

which recently has been shown to possess peptidoglycan hydrolase activity (53).

ORFA could encode a protein of 94 amino acids with a molecular size of 11,287 Da. The ORFA product has a homology of 64% (19% identity) with an 11.2-kDa hypothetical protein in the *rfaH-fre* intergenic region of the *E. coli* chromosome (2, 8).

Detection of *acmA* and its product in other lactococcal strains. *L. lactis* subsp. *lactis* IL1403, *L. lactis* subsp. *lactis* bv. diacetylactis 18-16S, and the *L. lactis* subsp. *cremoris* strains AM1, HP, and MG1363 were grown in GM17 for 36 h. Cell extracts were prepared and analyzed by renaturing SDS-PAGE with *M. lysodeikticus* cells as a substrate. Major bands of lytic activity at positions corresponding to sizes of 41 and 46 kDa were detected in all strains (results not shown). This result was complemented by PCR with the two sequencing primers PALA-4 and PALA-14 (Fig. 4), and the data are shown in Fig. 7. The same 1,131-bp DNA fragment was amplified from the chromosomal DNA of all the strains. These results indicate that all strains contain the same major peptidoglycan hydrolase activity which, in strain AM1, has been identified as an *N*-acetylmuramidase (32).

Construction and analyses of a chromosomal *acmA* deletion mutant. To investigate the function of AcmA, a deletion was introduced in the chromosomal copy of *acmA* by replacement recombination (28, 30). After transformation of *L. lactis*

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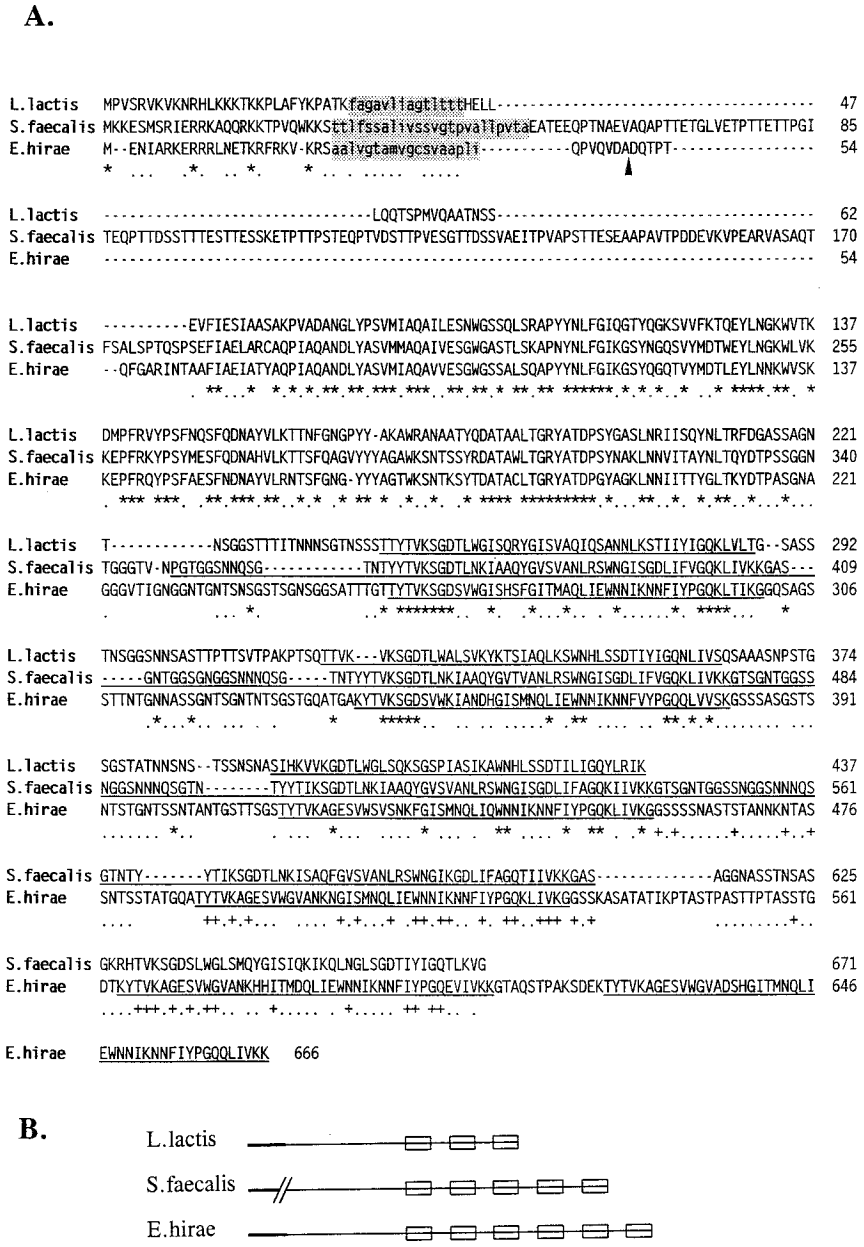

FIG. 5. (A.) Alignment of deduced amino acid sequences of AcmA of *L. lactis*, autolysin of *S. faecalis*, and muramidase-2 of *E. hirae*. The internally repeated regions of the three proteins are underlined. *, identical amino acids in all three proteins; +, identical amino acids between muramidase-2 and the autolysin of *S. faecalis* in the C termini of the two enzymes not present in AcmA; ·, similar amino acids; ▲, signal peptide cleavage site in muramidase-2; shaded and lowercase letters, putative membrane-spanning domains. (B) Schematic presentation of alignment shown in panel A. Thick line, (putative) signal sequence. The two slashes indicate that the sequence in the *S. faecalis* autolysin is not present in AcmA and muramidase-2. Repeated sequences according to the consensus proposed by Joris et al. (21) are boxed.

MG1363 with pINTAA, which carries *acmA* with an internal deletion, the integrants (Em^r and β-galactosidase producing) were checked by PCR with the primers PALA-19 and BK05AL (Fig. 8, labelled x and y, respectively). Integration via portions 1 and 2 of the insert of pINTAA resulted in PCR products with lengths of 2,550 and 3,247 bp, respectively. Three-quarters of the integrants obtained resulted from integration via portion 1 (results not shown). One of these integrants was used for further experiments. Excision of the integrated plasmid was established after nonselective growth for 30 to 35 generations in GM17. Cells were plated on GM17 plates containing X-Gal and 0.2% (wt/vol) autoclaved, lyophilized *M. lysodeikticus* cells and screened for loss of blue staining and halo formation. The chromosomal DNAs of two such colonies were analyzed by Southern hybridization (results not shown) and shown to have lost the *Hae*III site located between the *Sac*I and *Spe*I sites in

FIG. 6. Amino acid sequence alignment of carboxy-terminal repeats of AcmA (boxed) plus preceding and intervening sequences. The consensus sequence proposed by Joris et al. (21) is shaded. *, identical amino acids; ·, similar amino acids.

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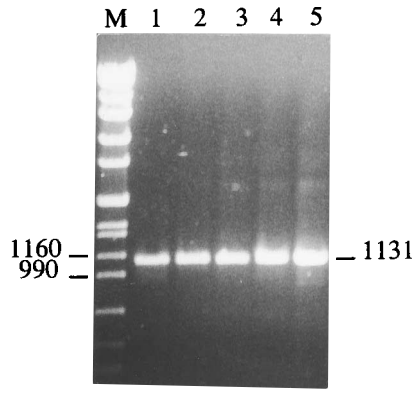

FIG. 7. PCR products formed on chromosomal DNA of lactococcal strains MG1363 (lane 1), IL1403 (lane 2), 18-16s (lane 3), AM1 (lane 4), and HP (lane 5). The primers used were PALA-4 and PALA14 (Fig. 4). Lane M, molecular weight markers (bacteriophage *Spp*I DNA cut with *Eco*RI), sizes of which (in base pairs) are indicated on the left and right.

acmA (Fig. 8). This result indicates that *acmA* is not an essential gene in *L. lactis.*

Cell extracts and supernatant fractions of the two deletion mutants and MG1363 grown in whey-based medium or GM17 were analyzed on a renaturing SDS-polyacrylamide gel containing autoclaved *M. lysodeikticus* cells as a substrate. Figure 9 shows that in both deletion mutants, no cell wall hydrolase activity was present, either in the cell extracts or in the supernatant fractions of GM17-grown cells. Also, after growth in whey-based medium, no clearing bands could be detected. The same results were obtained when autoclaved *L. lactis* cells were used as a substrate (results not shown). The fact that all the clearing bands normally present in cell extracts and supernatant fractions of *L. lactis* (Fig. 1) disappeared after inactivation of *acmA* indicates that all originated from AcmA.

As a result of the deletion of *acmA*, and in contrast to the wild-type strain MG1363, MG1363*acmAΔ1* did not lyse during prolonged stationary-phase growth, as measured by the reduction of the optical density (results not shown). After overnight growth in both GM17 and whey-based medium, sedimentation of MG1363*acmAΔ1* was observed. The cells of MG1363 and

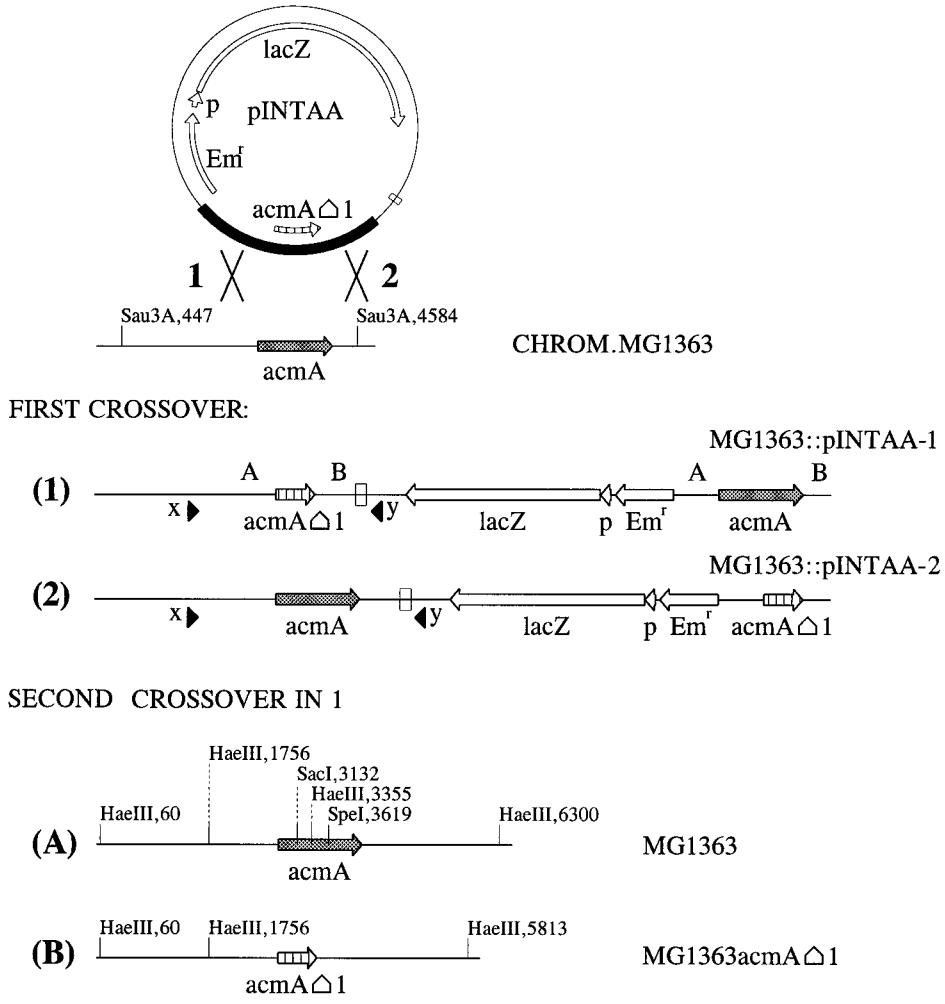

FIG. 8. Schematic representation of construction of chromosomal *acmAΔ1* mutant of *L. lactis* with pINTAA. Closed bar, insert of pAL09 in pORI280; *acmAΔ1*, deletion derivative of *acmA*; Em^r, erythromycin resistance gene; lacZ, β-galactosidase gene of *E. coli* expressed under control of lactococcal promoter P_{32} (p); open square, origin of replication of lactococcal plasmid pWV01; 1 and 2, possible sites for the first crossover; x and y, primers PALA-19 and BK05AL, respectively, used to distinguish between two types of Campbell integrants (labelled 1 and 2); A and B, possible regions for a second crossover and their products (labelled A and B). A number of relevant restriction enzyme sites are also shown.

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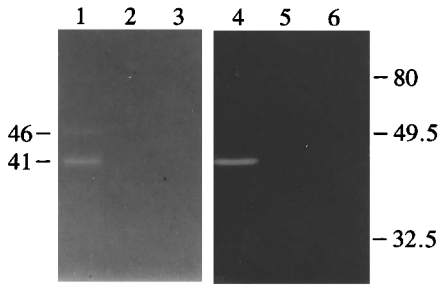

FIG. 9. AcmA activity in *L. lactis* MG1363 (lanes 1 and 4) and deletion mutants MG1363*acmAΔ1*-a (lanes 2 and 5) and *acmAΔ1*-b (lanes 3 and 6) by renaturing SDS–12.5% PAGE. The gel contained 0.2% (wt/vol) *M. lysodeikticus* autoclaved cells. Cell extracts (lanes 1 to 3) and supernatant fractions (lanes 4 to 6) after growth in GM17 were applied. The amount of sample was equalized according to the optical density of the cultures. Molecular masses (in kilodaltons) of standard proteins and of mature AcmA (46) or its secreted form (41) are shown on the right and left, respectively.

MG1363*acmAΔ1* were examined by light microscopy. MG1363*acmAΔ1* formed very long chains compared with MG1363 (Fig. 10). The addition of the culture supernatant of a wild-type strain to a culture of MG1363*acmAΔ1* dispersed the long chains (results not shown). These data indicate that the major lactococcal peptidoglycan hydrolase, AcmA, is needed for cell separation.

The data presented above conclusively show that *L. lactis* contains only one major peptidoglycan hydrolase, an *N*-acetylmuramidase (lysozyme) needed for cell separation.

DISCUSSION

In this report we present the cloning of the first peptidoglycan hydrolase of the genome of a lactic acid bacterium. The gene encodes the major peptidoglycan hydrolase of *L. lactis*. By PCR and denaturing SDS-PAGE, the gene was detected in all *L. lactis* strains used and, in fact, in all strains tested so far (unpublished observation). Our own unpublished results for strain MG1363 agree with the data for strain AM1, in which Mou et al. (32) observed only muramidase activity in the cell wall fraction. Because of these observations, together with the results of the homology studies presented here, we conclude that the cloned gene encodes the lactococcal *N*-acetylmuramidase, an enzyme hydrolyzing the linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine moieties. Accordingly, the gene was designated *acmA*. In a standardized assay to detect autolytic activity in a denaturing polyacrylamide gel, several lytic bands were found in both the cell and supernatant fractions of an *L. lactis* culture with *M. lysodeikticus* cell walls as a substrate, but only a few of these bands were found when cell walls of the host were used. This result indicates that the lytic activities of *L. lactis* are far more detectable with *M. lysodeikticus* cell walls as a substrate. This was also observed by Leclerc and Asselin (27) when they analyzed bacterial extracts of *Clostridium perfringens*, *Bacillus megaterium*, and *S. faecalis*. Comparison of the cell and supernatant fractions of the wild-type lactococcal strain and an *acmA* deletion mutant revealed that all the lytic bands present in the former originated from AcmA, as all disappeared in the deletion mutant. Because the smallest active band corresponded to a molecular size of approximately 29 kDa, a large part of AcmA can be removed without major loss of activity. The active site resides, most probably, in the N terminus (see below), and the deletions are thought to occur in the C-terminal repeated region. Degradation of cell wall hydrolases, without loss of activity, has been observed previously in *Bacillus licheniformis* (35) and *Bacillus subtilis* (23, 24, 38). In cell extracts of *E. coli* expressing *acmA*, two bands with sizes of 46 and 41 kDa and several smaller bands were present upon renaturing SDS-PAGE with cell walls of *M. lysodeikticus*. Bands with sizes of 41 and 46 kDa were also detected in cell extract of *L. lactis*, but only the smaller of the two was found in the supernatant fraction of *L. lactis*. The calculated molecular mass of AcmA (46,564 Da) corresponds to the size of the largest clearing band in the lactococcal cell extract (46 kDa). The n-region of the putative signal sequence of AcmA consists of 29 amino acids with 12 charged amino acid residues instead of the 8 to 12 generally observed in signal peptides of gram-positive bacteria (51). The signal sequence of muramidase-2 of *E. hirae* has 11 charged amino acids within the first 29 amino acids (7). On the basis of a signal peptide of 57 amino acids (50), the molecular mass of mature AcmA would be 40,264 Da. Most probably, AcmA is produced as a preprotein, and the secreted form is the 41-kDa protein that forms the major clearing band in the culture supernatant of *L. lactis*. The 46-kDa preprotein is either present in smaller amounts or has reduced activity in the assay used, as the corresponding band of activity is always less clear. The 41-kDa protein present in the cell extract is, most likely, the enzyme that is still attached with its C-terminal repeat region (see below) to the whole cell. The identity of the deduced amino acid sequences of AcmA, muramidase-2 of *E. hirae* (7), and the autolysin of *S. faecalis* (3) is especially high in the N-terminal regions of the three proteins which, most probably, encompass the active site (21). Within this region, homology to the flagellar protein FlgJ of *S. typhi-*

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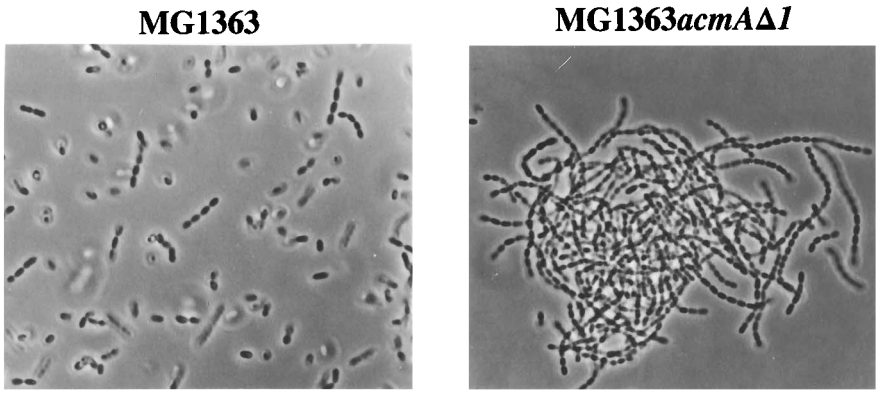

FIG. 10. Light microscopic view of *L. lactis* MG1363 (left) and MG1363*acmAΔ1* (right). Both strains were grown overnight in GM17. Magnification, ×1,000.

murium was also found (20). In the C terminus of AcmA, three repeated regions that conform to a consensus sequence are present, as postulated by Joris et al. (21). From the homology comparison presented here, it is clear that AcmA, muramidase-2, and the autolysin of *S. faecalis* contain three, six, and five such consensus sequences, respectively. All three cell wall hydrolases have a repeat at the extreme C terminus. Similar repeats have also been detected in the *B. subtilis* ϕPZA lysozyme, the homologous *Bacillus* gene 15 lysozyme, *L. monocytogenes* pathogenicity-associated protein p60, and *Staphylococcus aureus* protein A by Joris et al. (21) and in the sporulation-related τ-D-glutamyl-(L)*meso*-diaminopimelic-acid-hydrolyzing peptidase I of *Bacillus sphaericus* by Hourdou et al. (17). We also found this consensus twice in the N-terminal part of *E. coli* DniR, a protein affecting the anaerobic expression of hexaheme nitrite reductase (22), and twice in the C-terminal part of the lysine of the lactococcal bacteriophage Tuc2009 (1). The repeated regions are thought to be involved in substrate recognition and, thus, cell wall binding (21).

The *acmA* deletion mutant grows in long chains, causing settling of the culture after overnight growth. This result proves that AcmA is involved in cell separation as has been postulated previously (26, 31). The p60 protein of *L. monocytogenes* (53) and muramidase-2 of *E. hirae* (9) also have an essential role in cell separation. Further analysis of the deletion mutant will reveal whether *acmA* is the only lactococcal gene encoding a peptidoglycan hydrolase or whether *L. lactis* contains a second muramidase, as is the case in *E. hirae* (43). No halos were present when the deletion mutant was plated on GM17 containing *M. lysodeikticus* or *L. lactis* cell walls. Also, in an in vivo assay, MG1363*acmAΔ1* did not autolyze during prolonged stationary-phase growth. These results indicate that *L. lactis* does not express hydrolases that would have been missed in the denaturing SDS-PAGE procedure used here.

In the supernatant of an *L. lactis* culture, no protein corresponding to the lytic bands observed in an activity gel could be detected by SDS-PAGE. No differences between the protein banding pattern of the AcmA mutant and the wild-type strain by SDS-PAGE were observed (unpublished observation). These results suggest that the level of expression of the cell wall hydrolase is very low. Moreover, the activity may be regulated by proteolytic degradation, as has been observed in *B. subtilis* (19). Both phenomena are the subject of current research.

ACKNOWLEDGMENTS

We thank Anne de Jong for advice and support on the computer work and Henk Mulder for preparing of the photographs.

This work was supported by Unilever Research, Vlaardingen, The Netherlands. J.K. was the recipient of a fellowship of the Royal Netherlands Academy of Arts and Sciences (KNAW).

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